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In addition to accomplish the main goal of this grant proposal, i.e. to generating mice with estrogen receptor (ER) alpha gene ablation in the mammary gland of mcie, we have also carried out other tasks. A summary of our findings follows: (1) In order to identify targets of ER-alpha, ER-beta genes, we used microarray and profiled the targets for estrogen and bis phenol-A. This will be of help in understanding the mechanism by which different ligands function in the breast cancer cells. (2) We identified the F-domain of ER-alpha as playing an important role in the regulation of ER activity. (3) We constructed ER-alpha targeting vectors and generated ER-alpha embryonic cell lines. (4) We generated floxed ER-alpha mice, which will be used to generate tissue specific mice (e.g. mammary). Thus these tools and results will be of great use in understanding breast cancer disease process with reference to the role of estrogen receptor.

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Table of Contents

INTRODUCTION

Breast cancer is among the most devastating diseases affecting women. The risk for a North American women getting breast cancer has doubled since 1940, and at present one woman in eight is at risk of developing the disease. It is generally believed that estrogens play a significant role in the development of breast cancer. The mitogenic action of estrogen is amplified by the hormone-dependent transcription factors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). Because of its pivotal role in the normal physiology of breast, ERα has become a target for pharmacological intervention in breast cancer. This contention is strengthened by observations that the estrogen receptors are hormone dependent transcription factors that regulate the expression of growth factors and protooncogenes in breast tumor cell lines. Moreover, the growth and progression of many breast cancers are dependent upon estrogen, making measurement of ERα standard in the treatment decisions for patients with breast cancer. The ERα negative breast tumors are generally associated with faster growing and more aggressive tumors than the ERα positive tumors, which can be controlled with antiestrogen therapy. Towards understanding the role of ERα in breast tumorigenesis, we have initiated a program, using gene-targeting technology to ablate ERα gene in the mammary gland. This mouse model is expected to be of great use in addressing the role of estrogen receptor in mammary tumorigenesis.

In the final Progress Report we will describe our work with ERα mutant (K303R); yeast two-hybrid studies with ERα /ERβ heterodimerization; non-genomic action of estradiol and our accomplishments related to the main focus of the DOD-funded project, i.e. generation of conditional ERα knock out (KO) mice.

BODY

1. Study on human ER-K303R mutant:

We have used the cDNA encoding ER (K303R) to stably transfect an ER null cell line called C4-12, using the G418 selection system. The stable cell line was tested for expression of the transgene by Western blot analysis.

2. Protein-protein interactions between ERα and ERβ.

We have expanded the yeast two-hybrid experiments to study the interaction between ERs and the receptor coactivators and concluded that the F-domain of the receptor affects the estrogen-dependent interactions between p160 coactivators and the estrogen – bound ERs.

3. Studies with C4-12 breast cancer cells.

In this study, our most exciting achievement has been the establishment of breast cancer cell lines expressing ERα, ERβ, K303R or ERα/β. We have now systematically begun to analyze these cell lines to determine the target genes for ERs. In the first set of such an experiments, we challenged the ERα expressing cell line with vehicle, estradiol or an environmental estrogen bis phenol-A (BPA) and analyzed the gene expression pattern with microarray technology. The

intriguing observation is that some genes that are induced by estradiol are actually inhibited by BPA and the two ligands showed a different expression patterns. We have further confirmed the expression of some of these genes by real time PCR and focused on one of the protooncogenes, c-myb whose expression has been previously seen in breast cancer patients. We have cloned and sequenced its promoter, which revealed no consensus estrogen response element (ERE). Our current hypothesis is that C-myb is induced by estradiol and BPA through AP-1 elements on c-myb promoter.

4. Estrogen receptor-α gene-targeting vector and ES cell lines:
We have finished the construction of the ERα targeting vector and produced ES cell lines. The details are provided below:

Construction & characterization of the ER*α* gene-targeting vector.

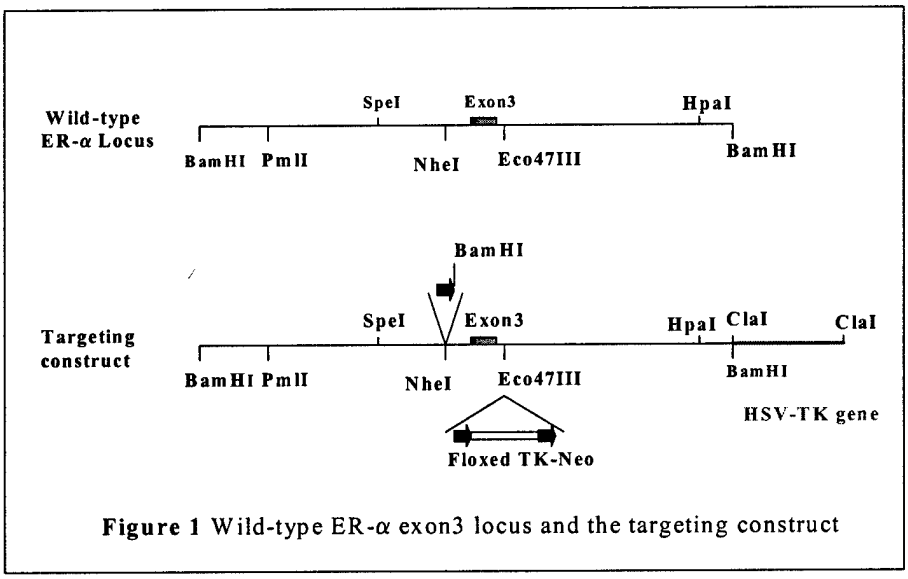

Figure 1 Wild-type ER-α exon3 locus and the targeting construct

In order to make the targeting vector, we originally focused to delete exon 2 and obtained a mouse genomic DNA clone harboring a 10kb BamHI fragment of mouse ER*α* gene. We however revisited the issue and decided to focus on deleting exon 3. This was prompted by the fact that knockout mice carrying exon 2 deletion in the genomic KO (reported by Ken Korach) some low levels of truncated ER*α* expression persisted.

We obtained the exon 3 DNA fragment (pBC-ER*α*-BamHI) from Jan Ake Gustafsson (Karolinska Institute). The pBC-ER*α*-BamHI construct was amplified and analyzed by restriction enzyme digestion and sequencing (fig. 1). We have confirmed that the 9.2 kb insert is the ER*α* genomic fragment and harbors exon3 (fig. 2).

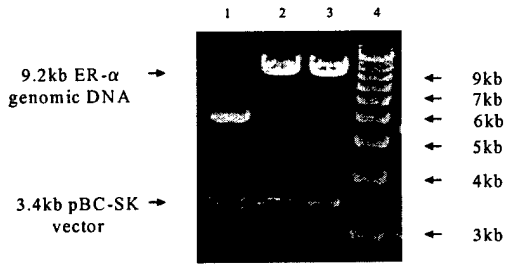

Figure 2 Confirmation of the ERα 9.2kb genomic DNA with restriction digestion. Lane 1: BamHI and SpeI digestion. Lane 2: BamHI digestion. Lane 3: BamHI and NotI digestion. Lane 4: 1kb marker (Promega).

Utilizing a series of cloning steps, we designed the ER*α* conditional Knockout-targeting construct according to a scheme presented in Figure1. In order to screen the targeted ES cell clones more efficiently, we introduced a negative selection gene, HSV TK, into the ClaI site of pBCSK vector (Stratagene). The "Floxed PGK-Neo cassette" was inserted into the Eco47III site on the 3' side of exon3. The 46bp LoxP-BamHI fragment was inserted into the NheI site on the 5' side of exon3. The targeting construct was confirmed by BamHI Digestion (fig 3) and DNA sequencing. The complete sequence of the 12.6 kb targeting construct was fully sequenced. We then blasted our DNA sequence in the Celera gene and found 97% homology with the Celera mouse genome. At this stage we decided to electroporate the targeting construct into the ES cells.

Electroporation of the targeting construct into ES cells.

The KO construct was linearized with PmlI and electroporated into embryonic stem (ES) cells. Most ES cells were killed by G418 treatment, but 30 single clones were obtained after the selection with G418 at a concentration of 240μg/ml. The ES clones were cultured in 24-well plates to full confluence and lysed overnight for the isolation of their genomic DNA. 5' and 3' PCR primers were designed to detect the targeted allele (fig. 4). The ES clones have been screened with ERKO 3' and 5' primers to detect the 5.2kb and 4.7 kb targeted alleles, but our PCR screening did not find targeted clones. The reason that most ES cells were killed was because of the reduced G418 resistance, resulting from a natural mutation in the Neo gene on our construct.

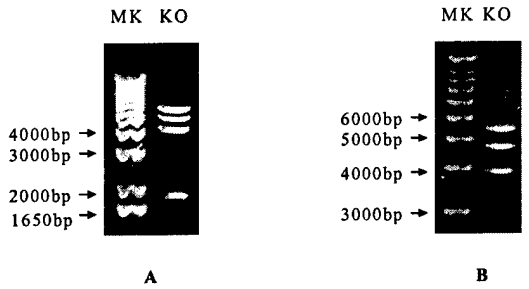

Figure 3 ERKO construct was confirmed with BamHI digestion. Two BamHI sites have been introduced into the KO construct at LoxP-BamHI site and the 3' end of Floxed TK-Neo cassette. 4 bands are generated by BamHI (1.9kb, 3.9kb, 4.7kb and 5.4kb). Panel B is a longer run of Panel A. Promega 1kb marker (MK) was used. Lane 2 shows KO construct BamHI digestion.

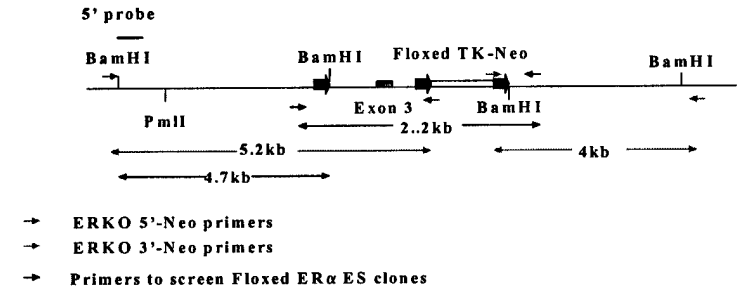

Figure 4 Strategies to screen and confirm the targeted allele and Floxed ERα alleles. The ES clones will be Screened with ERKO 5'-Neo and ERKO 3'-Neo primers for the targeted alleles. The targeted allele will be confirmed with Southern blot using 5' probe.

Repairing the Neo gene on the targeting construct

We decided to fix the mutation in our knockout construct with site-directed mutagenesis. The mutation was repaired on the pFloxNeo plasmid. The mutant Neo on the KO construct was then replaced with the fixed Neo gene. The KO construct was then linearized with PmlI and electroporated into ES cells again.

Screening of targeted ES clones

After electroporation, about 290 ES clones were amplified after the G418 selection. Genomic DNA was extracted from the lysate of the ES cells. Two sets of PCR primers, ERKO 5'-Neo and ERKO 3'-Neo, were used to screen the targeted ES clones. Both 5' and 3' primers are from flanking region of the targeting locus (fig. 4). 3 positive clones were identified by PCR (figure 5).

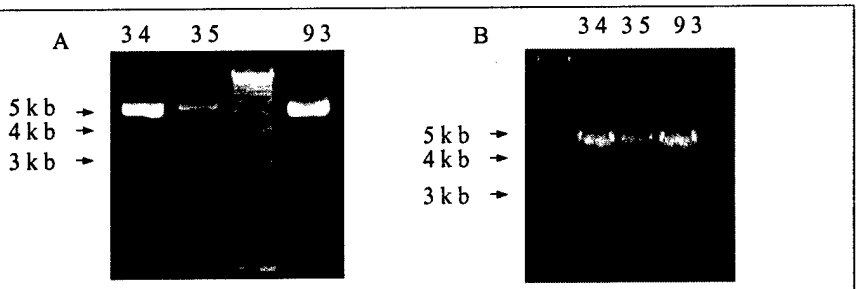

Figure 5 Identification of the targeted ES clones by 5' and 3' PCR . PCR has been done with the 5' and 3' outside primers shown on Figure 4 to detect a 5.2kb 5' fragment and 4.7kb 3' fragment from the targeted alleles. 3 positive clones, D34, D35 and D93, were identified in our screening. Panel A showed the 5' PCR result panel B is the 3' PCR result.

For confirmation, the 5' and 3' PCR products of the positive clones were subjected to Southern Blot with 3' and 5' outside probes. The PCR Southern experiment confirmed that the PCR products are from the targeted alleles (figure 6).

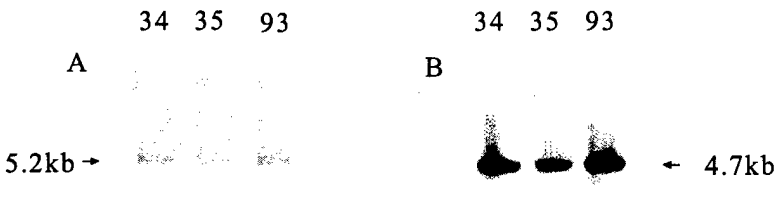

Figure 6 Confirmation of the targeted clones with Southern Blot of the 5' and 3' PCR products. ERα 5' and 3' outside probes have been used to confirm the 3' and 5' PCR results. The Southern results show that the PCR products are from the targeted alleles. Panel A showed the 5' PCR Southern. Panel B showed the 3' PCR Southern.

Genomic DNA from the positive clones was digested with BamHI and probed with 5' outside probe, a 1.1 kb DNA fragment generated with NotI and PmlI

double digestion on the ERKO construct. Two positive clones, D34 and D93, were re-confirmed by the Southern Blot (figure 7).

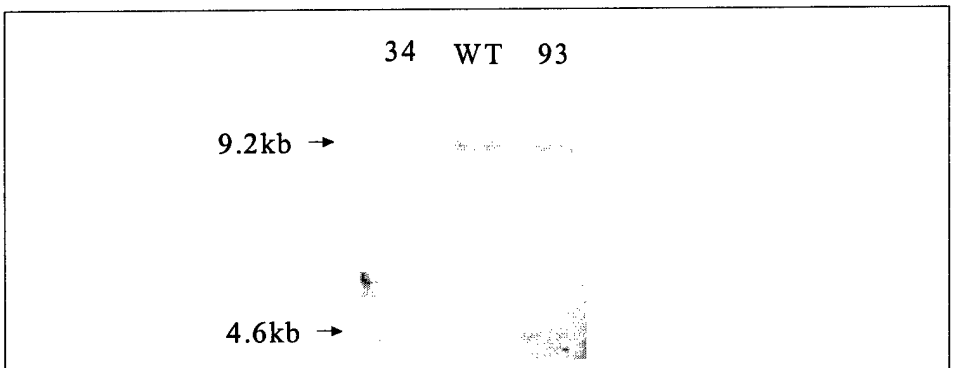

Figure 7 Confirmation of the targeted ES clones with Southern Blot. Genomic DNA of the targeted clones identified by the PCR screening was digested with BamHI and probed with the 1.1 kb 5' outside probe. The wild type ES cells (WT) showed 9.2kb bands. The target ES clones, 34 and 93, showed 4.6kb targeted allele bands in addition to the 9.2 kb wild type bands.

Identification of the Floxed ERα ES clones

It has been reported that insertion of a Neo gene will affect the phenotype of knockout mice. We decide to delete the Neo gene in our Targeted ES clones. The Cre recombinase plasmid was electroporated into the mixed targeted clones (D34 and D93) to delete the Floxed Neo gene. We got 30 G418 sensitive clones from the selection. The clones were screened with ERKO 5'-Neo and ERKO 3'-Neo primers. No PCR product can be amplified from the 30 clones. This result confirmed that the Neo gene has been deleted from our ES clones. Since there are three possible recombinations by the Cre treatment, these negative clones were further screened with primers that amplify the region between LoxP1 and LoxP3. 1050bp fragment will be amplified if only the Floxed Neo gene is deleted. A 500bp DNA fragment will be amplified if both exon 3 and the Floxed Neo are deleted by Cre recombinase. Since only one allele of a ES clone was expected to be targeted, we will see the 970bp wild type allele band in all the ES clones. As the Floxed Neo gene was deleted in all the clones we did not see the 1.7 kb band resulting from the recombination of LoxP1 and LoxP2. We found 3 Floxed ERα ES clones in our screen (Figure 8). One of the 3 identified clones, E84, was confirmed with Southern blot using 3' probe (Figure 9).

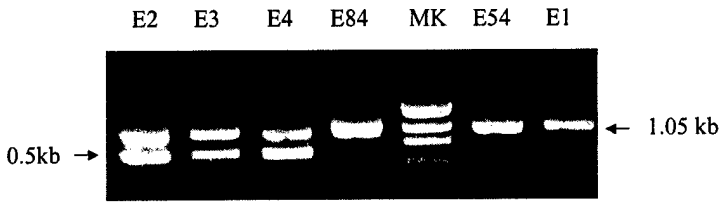

Figure 8 Identification of the Floxed ERα ES clones with PCR. The targeted ES clone 93 was transiently transfected with Cre recombinase construct to delete the Floxed PGK-Neo gene. The genomic DNA of G418 sensitive clones was amplified and subjected to PCR with the primers amplifying the Floxed ERα exon 3 region. The LoxP1 and LoxP3 recombination, which delete the PGK-Neo and the ER exon 3, generated ~0.5kb band. The 1.05kb band represent the LoxP2 and LoxP3 recombination which delete the Floxed PGK-Neo gene. The 1.05 kb Floxed ERα allele and the 0.97kb wild type allele are shown as one band in E84, E54 and E1. E2, E3 and E4 showed 0.97kb wild type band and the 0.5kb band. The Low Mass DNA ladder (Invitrogen) was used as DNA marker (MK).

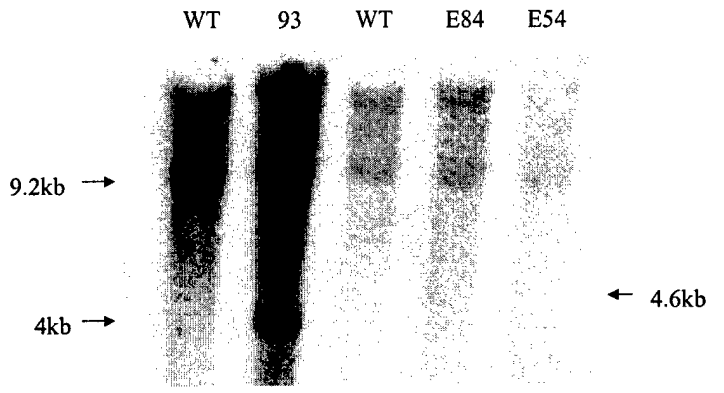

Figure 9 Floxed ERα ES clone is confirmed with Southern Blot. The genomic DNA of the Floxed ERα clones was digested with BamHI and probed with the ERα 3' probe. All samples showed the 9.2kb DNA fragment of the wild type allele. Targeted ES clone, 93, showed the 4kb fragment of the targeted allele. Floxed ERα clone, E84, showed the 4.6 kb Floxed ERα allele, resulting from the deletion of the BamHI site in the Floxed Neo gene.

Generation of the Floxed ERα mice

The ES cells from clone E84 were introduced into pseudo-pregnant mice. Four agouti males were born. Breeding with black 6 females, a 95% agouti chimera generated 4 agouti pups in the second litter. Genotyping the mouse-tails showed that 3 of the 4 agoutis are Floxed ERα mice (Figure 10). We are breeding the

Floxed ERα mice to generate the homozygote. Crossing the Floxed ERα mice with WAP-Cre mice will generate the MERKO mice.

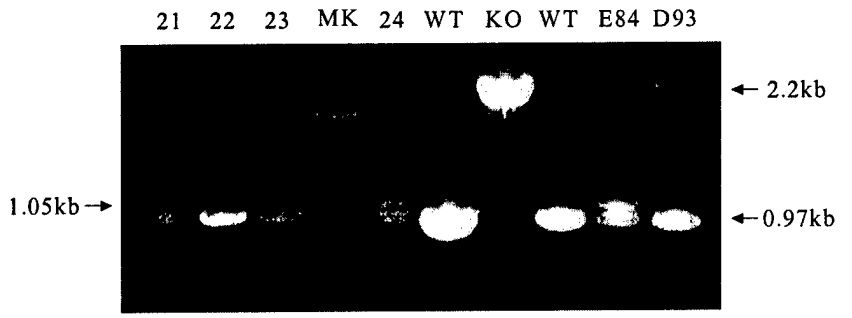

Figure 10 Confirmation of the Floxed ERα mice with PCR. The genomic DNA from tails of agouti mice was extracted and subjected to PCR with the primers amplifying the Floxed ERα exon 3 region. The wild type mice showed the 0.97kb wild type alleles. The Floxed ERα mice (21,23 and 24) and Floxed ERα ES clone, E84, showed the 0.97kb wild type allele and the 1.05kb Floxed ERα allele.The ERKO plasmid (KO) and Targeted ES clone, D93, showed the 2.2kb DNA containing the Floxed PGK-Neo gene. The Low mass DNA ladder (Invitrogen) was used as DNA marker (MK).

Key Research Accomplishments:

- Identification of the target genes of estrogen and BPA in breast cancer cell line expressing only ERα.
- Contribution to the F-domain of ERα in the estrogen-dependent recruitment of coactivators.
- Construction of the ERα targeting vector and generation of Floxed ERα ES cell lines.
- Generation of Floxed ERα mice for generating mammary epithelial specific ERα KO mice.

REPORTABLE OUTCOMES

Singleton, D., and Khan, S. "Estrogen receptor dependence of MAP-kinase activation in breast and uterine tumor cells Annual Meeting of the "ENDOCRINE SOCIETY", Denver, Co (2001)

Singleton DW, Feng Y, Burd C, Khan SA. Nongenomic activity and subsequent c-fos induction by estrogen receptor ligands are not sufficient to promote deoxyribonucleic acid synthesis in human endometrial adenocarcinoma cells. Endocrinology. 2003 Jan;144(1):121-8.

Singleton DW and Khan SA. Xenoestrogen exposure and mechanisms of endocrine disruption. Front Biosci. 2003 Jan 1;8:s110-8. Review.

Singleton DW, Feng Y, Burd C, Khan SA. Nongenomic Action of Estrogen Receptor Ligands Is not Sufficient to Induce Proliferation of Human Endometrial Adenocarcinoma Cells. (abstract submitted for presentation at The Endocrine Society Meeting, June 2002)

Feng Y, Singleton D and Khan S. Conditional Estrogen Receptor Knockout Mouse Model For Studying Mammary Tumorigenesis. (abstract submitted for presentation at The Era of Hope Meeting, September 2002)

David W. Singleton, Yuxin Feng, Yangde Chen, Steve J. Busch, Adrian V. Lee, Alvaro Puga and Sohaib A. Khan. Bisphenol-A and Estradiol Exert Novel Gene Regulation in a Human Breast Cancer Cell Line (In revision for Molecular and Cellular Endocrinology)

Jun Yang, David W. Singleton, Elwood V. Jensen and Sohaib A. Khan. The F-domain of Estrogen Receptor-Alpha Inhibits Ligand Induced Receptor Dimerization. (preparing for submission to the Proc. Natl. Acad. Sci.)

Cell Lines:

We have generated yeast cell lines transformed with different pairs of the estrogen receptor.

CONCLUSIONS

Estrogen receptor *α* plays a key role in breast cancer. However, little is known about the involvement of the receptor in the progression of breast tumorigenesis. Towards achieving our overall goal of generating ER knockout mice for studying mammary tumorigenesis, we had proposed to generate ERα conditional knockout mice. We have successfully generated ERα targeting construct, Floxed ERα ES cell line and the Floxed ER*α* mice for use in generating ERα knockout mice. We are breeding the Floxed ER*α* mice with WAP-Cre mice. MERKO mice will be generated in several months. In addition, we established breast cancer cell lines expressing different ERs; identified novel ER*α* target genes regulated by E2 and BPA and defined the function of F domain of ER*α* in coactivator recruitment.

Our mammary gland specific ERα knockout project is highly innovative in that no mouse model is available where the ER gene is only inactivated in the mammary epithelial cells. The conditional gene knockout is a useful advancement in the field and its use in understanding issues related to breast cancer will have tremendous advantages. We will use the mouse model to determine if the mammary gland functions in the absence of estrogen receptor and whether mammary tumors can be induced by DMBA in ER*α* negative mice. In the future, using a transgenic approach, we will reintroduce variant ERs into the MERKO mice. These mice will then express defective ER only in the mammary cells and

can be useful to determine the consequence of aberrant ER expression on tumor development.

The generation of Floxed ERα mice provides a pivotal animal model, not only important to study the role of ERα in breast tumorigenesis, but also useful to study the biological functions of ERα in other organs, such as brain and heart. Crossing the Floxed ERα mice with tissue specific expressing Cre mice, tissue specific ERα knockout mice in ERα effective organs, brain, heart, bone and prostate, can be generated.

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